MOLECULAR REPRODUCTION AND DEVELOPMENT 52:50–56 (1999)

Improved Flow Cytometric Sorting of X- and Y-Chromosome Bearing Sperm: Substantial Increase in Yield of Sexed Semen

WIM RENS, GLENN R. WELCH, AND LAWRENCE A. JOHNSON*

Germplasm and Gamete Physiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland

ABSTRACT The yield of flow cytometric sorted X- and Y-chromosome-bearing sperm in a given time period is an important factor in the strategies used for fertilization and the production of sex-preselected offspring. This yield is dependent on the efficiency with which the modified flow cytometer/cell sorter analyzes the DNA of spermatozoa. The efficiency is directly related to the number of sperm with the correct orientation during DNA analysis. Currently, the efficiency of flow cytometric sperm sorting is low since orientation of the sperm head to laser excitation is rate limiting. To overcome this problem, a new nozzle was designed to enhance sperm orientation and tested under flow cytometric sorting conditions. The degree of orientation improvement was determined with different sample rates using viable sperm and dead sperm of several different species. There was at minimum, a two-fold increase in the proportion of oriented sperm when comparing the new nozzle with the currently used modified flow cytometer/cell sorter employing a beveled needle. More than 60% of intact bull sperm and boar sperm were correctly oriented compared with 25% to 30% using the beveled needle system. A unique characteristic of the novel nozzle was that the proportion of oriented sperm was independent of sample rate and of sperm motility. The accuracy of DNA measurement together with high purity sorting was tested using the novel nozzle. The novel nozzle was unique in that accuracy of measurement and sorting performance were not diminished. Using the new nozzle, samples of 88% purity of sorted X-sperm and Y-sperm were obtained for viable bull and boar sperm. The yield of flow cytometric sorted X- and Y-chromosome-bearing sperm using the novel nozzle was, on average, twice that obtained by using the beveled needle system in conjunction with a standard equipment nozzle for orientation. *Mol. Reprod. Dev. 52:50–56, 1999.* Published 1999 Wiley-Liss, Inc.†

Key Words: gender preselection; DNA analysis; nozzle; sorting

INTRODUCTION

The physical separation of semen into viable X- and Y-chromosome–bearing sperm populations using flow cytometry and sorting has proven to be effective for several species of domestic animals, including rabbits (Johnson et al., 1989), swine (Johnson 1991; Rath et al., 1997), cattle (Cran et al., 1995; Seidel et al., 1997), and sheep (Johnson 1992; Cran et al., 1997; Catt et al., 1997). Human offspring have also been born using this sexing procedure (Fugger et al., 1998. Sorting of viable sperm is performed with a modified flow cytometer/cell sorter (Johnson and Pinkel, 1986), using the DNA content of sperm as the discriminatory parameter. The success of the sorting process is dependent on the accuracy and efficiency of the sperm analysis for DNA. The precision of the DNA analysis is necessarily high (CV $<$ 1.2%) as the difference in DNA content is small (Pinkel et al., 1982; Johnson and Pinkel, 1986; Johnson, 1995). Consistent high resolution measurements can also be obtained by using the slit-scanning technique (Rens et al., 1996), however application for commercial sorting is not feasible.

One of the characteristics of sperm separated on the basis of DNA content using flow cytometric sorting is the relatively low numbers produced per unit of time ($\sim 3 \times 10^5$/hr; Johnson et al., 1989). To overcome the low numbers various alternatives to artificial insemination have been used. Surgical insemination has been used for producing offspring in swine, rabbits and sheep while in vitro embryo production has been used in cattle and swine while intra-cytoplasmic sperm injection (ICSI) has been used for sheep and cattle. Most recently deep uterine insemination was found effective for producing sexed offspring in cattle using ($\sim 2 \times 10^5$ sperm; Seidel et al., 1997). Numbers of sperm used in the various systems vary: surgical insemination (3×10^5 per oviduct; Johnson et al., 1989) in vitro fertilization (2×10^4 per egg; Cran et al., 1995) to 50–250 sperm per egg (Rath, et al., 1997; Long et al., 1998) ICSI using a single sperm (Catt et al., 1997; K Hamano, personal communication regarding cattle ICSI, 1998). The success of fertilization with any method with the exception of ICSI is influenced by the number of sperm available

W. Rens' current address: Cambridge University, Department of Pathology, Tennis Court Road, Cambridge, United Kingdom CB21QP.

*Correspondence to: L.A. Johnson, USDA, ARS, GGPL, B-200, BARC-East, Beltsville, MD 20705. E-mail: lajohnsn@ggpl.arsusda.gov

Received 18 June 1998; Accepted 21 July 1998

and the time interval between the start of the sorting process and the time sperm are delivered to the site of fertilization. The number of sperm available would be increased and the time interval decreased if it were possible to increase the sperm sort rate by increasing the efficiency of sperm analysis/sorting, e.g., increase the fraction of oriented sperm.

Improved efficiency of sperm head orientation is the key factor to increased yields of X and Y sorted sperm. Due to the sperm's flat ovoid shape and compactness of chromatin, only sperm with the proper orientation to the intersecting laser beam during detection can be effectively analyzed for DNA content of sperm heads (Johnson and Pinkel, 1986) and of intact sperm (Johnson et al., 1989). Increasing the proportion of sperm that have their edge properly aligned (oriented) to the 90° fluorescence detector of the sperm sorter is necessary for increased production of sorted sperm. Previously we have used a beveled needle on a commercial sorter (Johnson and Pinkel, 1986) to enhance the yield over random orientation. Characteristic orientation of intact sperm passing through the system is 20% to 30% (Johnson et al., 1989) using the beveled needle. Recently we have improved the proportion of sperm aligned with their edge to the 90° fluorescence detector by two to three times using a newly developed elliptical nozzle (Rens et al., 1998). The new nozzle is capable of moving the forces of orientation much closer to the exit orifice of the nozzle and thus maintaining proper orientation of a greater proportion (60% to 70%) of sperm as they pass the laser beam and thus discarding less sperm due to misorientation.

This paper describes the application of a new elliptical nozzle on a standard speed cell sorter modified for sorting sperm. The nozzle is designed to significantly improve sperm orientation during passage through the sperm sorter. The characteristics and performance of this new nozzle are investigated for sorting viable X- and Y-chromosome–bearing sperm, especially with regard to sperm orientation, DNA measurement and sorting efficiency.

MATERIALS AND METHODS

Sperm Preparation and Staining

Ejaculated semen from 15 mature bulls, three boars, and three rabbits, maintained on regular sperm production schedules, were used for this study. In addition, mouse and human semen samples were analyzed to assess the effect of differing head morphology on the improved nozzle design. Sperm preparation and staining were based on methods described by (Johnson et al., 1987, 1989; Johnson, 1991). Briefly, aliquots of neat semen were extended to a concentration of 15×10^6/ml in Hepes buffered medium containing 0.1% BSA (pH = 7.4) for bull sperm, Beltsville TS extender (BTS, pH = 7.2) for boar sperm, and Tris buffer (0.21 M Tris, 58 mM glucose, and 67 mM citric acid; pH = 6.9) for rabbit sperm (Johnson et al., 1989; Johnson, 1991). Mouse and human semen were also extended in BTS. Sperm were subsequently stained with 7.1 µM Hoechst 33342 (Calbiochem-Behring Corp., La Jolla, CA) and incubated over a 40-min period at 32°C. For the bull sperm studies, just prior to analysis, propidium iodide (1.5 µM, Calbiochem-Behring Corp.) was added to the Hoechst 33342 stained sperm. This allowed dead sperm to be distinguished from living sperm (Johnson et al., 1994). This removal of dead sperm improves sorting purity and efficiency. Stained sperm were also visually examined to determine the percentage of motile sperm using a Zeiss Axiophot fluorescence microscope (365 BP excitation filter and 420 LP filter for Hoechst 33342 fluorescence, 450–490 BP excitation filter and 520 LP filter for PI fluorescence; Carl Zeiss Inc., Thornwood, NY).

Flow Cytometry

Sorting sperm. An EPICS 750 series flow cytometer/cell sorter (Coulter Corp., Miami, FL) was previously modified for flow sorting sperm (Johnson and Pinkel, 1986) and used to separate viable X from Y sperm using the Beltsville Sperm Sexing Technology (Johnson et al., 1989). The modifications were designed to minimize the orientation artifact caused by differential emission of fluorescence from the edge of the sperm compared to the face of the sperm, and therefore to increase the accuracy of measurement of relative sperm DNA content. Briefly, the modifications consisted of the addition of a second fluorescence detector, in the forward position in addition to the standard right angle detector, and a beveled sample injection needle. The alternative system introduced in this paper used a novel nozzle as a replacement for the standard nozzle (Rens et al., 1998) in combination with a standard cylindrical needle rather than a beveled needle to orient sperm. The fluorochrome of the stained sperm was excited with ultraviolet light (UV, 351, 364 nm multi-line) from a 5W 90–5 Innova argon-ion laser (Coherent, Palo Alto, CA) operating at 175 mW. UV blocking filters (418 LP) were used in both fluorescence detectors. Approximately 100,000 sperm containing the X- or Y-chromosome were sorted (Johnson et al., 1989) into separate 0.65 ml micro (presiliconized) centrifuge tubes (single drop sorting). Sorted sperm samples were pulse sonicated to remove sperm tails and restained to approximately the original Hoechst 33342 concentration and reanalyzed on a second flow cytometer (EPICS V, Coulter Corp.) modified for sperm. Resulting DNA histograms were analyzed by fitting them to a pair of Gaussian distributions for purity determination (Johnson et al., 1987, 1989).

New orienting nozzle. The novel nozzle was based on the standard Coulter EPICS V/750 series nozzle (76 µm flow cell tip, catalog no. 6602836), but instead of a tapered circular, shaped interior, the inside of the nozzle had been formed into a tapered elliptical shape (Rens et al., 1998). In this way sperm were oriented deep into the nozzle. The sperm exited the nozzle, through a round 76 µm jeweled orifice into air and passed through the laser beam. The novel nozzle was

used in combination with a standard cylindrical sample injection needle (Coulter Corp.) to orient sperm.

Experimental Design

Several experiments were conducted to investigate the characteristics of the novel nozzle when applied for viable sperm sorting. First the performance of the new nozzle was compared with a standard flow cytometry system and with a modified system using a beveled needle (Experiment 1). Additionally the influence of sperm motility on orientation was analyzed (Experiment 2). Motility is an important feature for viable sperm sorting, as only motile sperm samples (>70%) are considered suitable for sexing and subsequent fertilization. Experiment 3 was designed to assess the influence of sample rate on orientation. Sample rate is important as it is advantageous to sort sperm in the shortest amount of time possible. In experiment four, sperm collected from rabbits, mice and humans were analyzed to investigate the orientation performance of the novel nozzle for these species due to their contrasting sperm morphology.

Experiment 1. Semen of eight different bulls was analyzed on two different days with the standard nozzle and standard cylindrical needle. Semen of 15 different bulls was analyzed on three different days and measured with the standard nozzle and beveled needle, and also with the new nozzle and standard cylindrical needle.

Sample rates were about 2,000 sperm per second. Window settings to select oriented sperm signals were the same for each experiment. Measurements of sperm with the standard and new nozzle were done with different cell sorters (an EPICS V and EPICS 750 series). In this way, low proportions of oriented sperm caused by changing needles back and forth was avoided.

Experiment 2. Measurements were performed with the orienting nozzle. Proportions of oriented intact viable sperm were compared with proportions of oriented sperm without their tails and proportions of oriented dead intact sperm. Measurements were performed on four different days with semen of different bulls. Sample rates were 2,000 sperm per second. Viable sperm could be analyzed separately from dead sperm by their differential Hoechst fluorescence, because PI, which only stains dead sperm partially, quenches Hoechst fluorescence thus forming a separate population of dead sperm (Johnson et al., 1994).

Experiment 3. Semen of eight bulls was measured on two days with sample rates of 500 per sec and 2,000 per sec.

Experiment 4. Semen of three different rabbits was analyzed with the orienting nozzle and proportions of correctly oriented sperm were determined. Additionally, mouse sperm and frozen-thawed human sperm were analyzed to demonstrate the use of this nozzle for these species.

Experiment 5. This experiment was performed to test the purity of the sperm sample sorted with the new nozzle. An improvement in orientation of sperm is only valuable for sperm sexing if (a) The orientation does not decrease the accuracy of DNA measurement and (b) sort stability is maintained. Sorting of X and Y bearing sperm was performed on four different days for bull sperm and three different days for boar sperm. Only samples with 70% or more motile sperm were used. Reanalysis was performed to determine the purity of the sorted sperm populations.

Statistical analysis was performed using Student's *t*-test for small samples (Steel and Torrie, 1960).

RESULTS

Experiment 1

A large improvement in bull sperm orientation was achieved using the novel nozzle compared with the standard nozzle fitted with a cylindrical or beveled needle (paired difference *t*-tests, $P < 0.05$, separate data not shown). On average, a 3.0 times larger proportion of oriented sperm was obtained with the novel nozzle (52.5 ± 4.7%, n = 15) when compared with standard nozzle and standard cylindrical needle (17.3 ± 0.7%, n = 8) and a 2.3 times larger proportion of oriented sperm when compared with the beveled needle (22.7 ± 3.5%, n = 15).

Experiment 2

Proportions of oriented viable bull sperm (56.8 ± 6.7%) were the same as those of tailless bull sperm (59.8 ± 4.2%) or dead bull sperm (53.3 ± 4.3%, no significant difference at $P = 0.05$, n = 4), showing that motility has a negligible influence on orientation of bull sperm when the novel nozzle is used.

Experiment 3

This experiment was performed to investigate the influence of sample rate on orientation. Orientations of 52.1 ± 6.4% and 52.3 ± 6.5% were obtained for sample rates of 500 per sec and 2,000 per sec, respectively (n = 8, paired difference *t*-test, $P = 0.05$, separate data not shown), demonstrating that orientation was not influenced by sample rate.

Experiment 4

A high proportion of rabbit sperm were correctly oriented when measured with the novel nozzle (48 ± 2.4% n = 3). The proportion of oriented sperm obtained with mouse sperm and human cryopreserved-thawed sperm was 44% and 45%, respectively.

Experiment 5

Excellent sort stability was maintained with the novel nozzle. Droplet streams as well as droplet delay were stable during sorting with recoveries over 90% using single drop deflection. Bull sperm orientation was on average 54.5% which is about the same as the results of experiments 1–3. High purity sorted samples were obtained on all four days of the experiment (Table 1). The mean for X sperm sort purity was 87.6% and for Y-sperm 89.3%.

TABLE 1. Sort Purities of Bovine X-Sperm and Y-Sperm Measured With the Novel Nozzle

Day	Orientation (%)	X-sort (%)	Y-sort (%)
1	50	88.0	90.5
2	57	91.5	90.5
3	59	84.0	85.5
4	52	87.0	90.5
Mean ± sd	54.5 ± 4.2	87.6 ± 3.1	89.3 ± 2.5

TABLE 2. Sort Purities of Porcine X-Sperm and Y-Sperm Measured With the Novel Nozzle

Day	Orientation	X-sort (%)	Y-sort (%)
1	61.5	84.0	89.0
2	60.0	84.3	87.3
3	60.0	87.7	92.0
Mean ± sd	60.5 ± 0.9	85.3 ± 2.1	89.4 ± 2.4

A three-day sorting experiment was also performed with boar sperm. The mean X sperm sort purity was 85.3% and the Y sperm sort purity was of 89.4% (Table 2). Proportions of oriented sperm averaged 60.5 ± 0.9%. Orientation histograms (90° fluorescence) obtained with the novel nozzle and the beveled needle system are presented in Fig. 1 show the significant improvement in orientation that can be achieved with boar sperm with the beveled needle and the new orienting nozzle.

DISCUSSION

The sorting of sperm into X- and Y-chromosome bearing populations with a flow cytometer/cell sorter is based on their differential DNA content. Sperm are stained with the DNA binding fluorochrome, Hoechst 33342 and the fluorescence is collected and measured. However, in order to precisely distinguish X and Y sperm it is necessary to analyze the forward fluorescence of only correctly oriented sperm i.e. sperm with their narrow edge directed toward the 90° fluorescence detector. The reason for this is that the fluorescence is not uniform due the flat ovoid shape of the sperm head and the compactness of its chromatin. Consequently, a brighter fluorescence is exhibited from the edge than flat side. In an unmodified flow cytometer/cell sorter, sperm traverse the laser beam in a random orientation resulting in little or no differential X-Y DNA analysis.

To improve analysis efficiency, the proportion of oriented sperm can be increased by using a beveled sample injection needle (Johnson and Pinkel, 1986; Johnson et al., 1989). The sample core leaving the beveled needle is in the shape of a ribbon, which applies orienting forces to the sperm. However this ribbon only exists when the sample stream is narrow (this means a low sample pressure causing a low sample rate), which is disadvantageous for efficient sperm sorting. Orientation of intact sperm is also more difficult than orientation of sperm heads (tailless sperm) due to the effect of the tail while in fluid motion (Johnson et al., 1989).

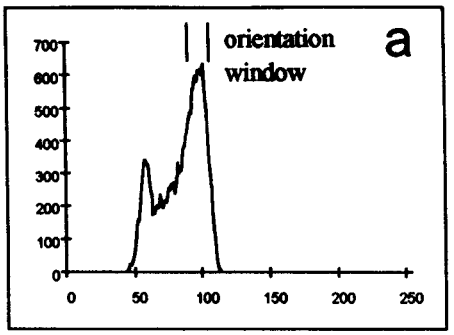
To overcome both problems, a novel nozzle was designed to orient sperm. The novel nozzle had the additional advantage that problems associated with placing a beveled needle in the right position were avoided, because the modified EPICS nozzle is screwed onto the flow cell providing the same nozzle position each time it is removed and replaced. The inside of the nozzle was modified such that sperm are subjected to orientation forces from the time they leave the injection needle until the time they enter the jewel and exit the orifice. To guide sperm to the slit, the inside of the nozzle had a specific asymmetric tapered elliptical shape (Rens et al., 1998). Orientating sperm in this way could induce several problems. A wider sample stream or turbulence could affect the accuracy of fluorescence analysis. Although the orifice of the jewel was still round, the slit could have been problematic for sort droplet formation. Therefore, together with studies to investigate improvement of orientation, sort studies were carried out to investigate these concerns, because the main purpose was the efficient sexing of sperm.

In the first series of experiments, orientation measurements with the new nozzle were compared with a conventional system: a standard nozzle and a standard sample injection needle (in a conventional system, sperm traverse the laser beam with random orientation). The difference in outcome between these two systems was a threefold increase in the proportion of oriented sperm. A two to three fold increase in efficiency was found when the new nozzle was compared with the beveled needle system. Experiment 2 showed that sperm motility had no influence on orientation of bovine sperm, with tailless sperm and dead intact sperm having the same proportions of oriented sperm as viable sperm. Thus the use of the novel nozzle should lead to less day to day variation in the percentage of oriented bovine sperm. At flow rates of 2,000 sperm per sec the new nozzle was clearly superior to the beveled needle in percentage of sperm oriented (54% and 23%) respectively. This translated to a sort rate of 200 sperm sorted in each direction for the novel nozzle, double that with the beveled needle.

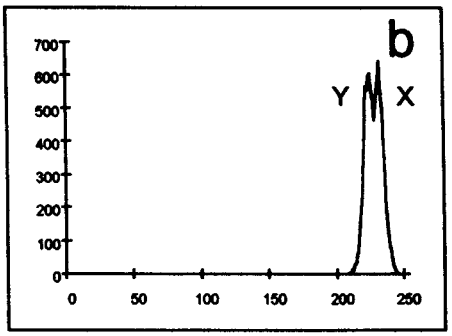
The influence of sample rate was tested in Experiment 3. A low sample rate (500 sperm per sec) resulted in the same proportion of oriented sperm as a high sample rate (2,000 sperm per sec). Initial experiments with even higher sample rates (3,000–4,000) gave similar results (data not shown). When a beveled needle is used to orient sperm the fraction of oriented sperm decreases with increasing sample rate. This means that the gain in proportion of oriented sperm is higher with higher sample rates which is beneficial for efficient sperm sorting.

The final experiments investigated the effect of the new nozzle on DNA analysis accuracy and sorting behavior. The effective separation of X- and Y-chromosome bearing sperm by sorting is a prerequisite for practical application of the new orienting nozzle. All samples were run with a sample rate of 2,000 per sec. Window settings were the same as in the control sorting

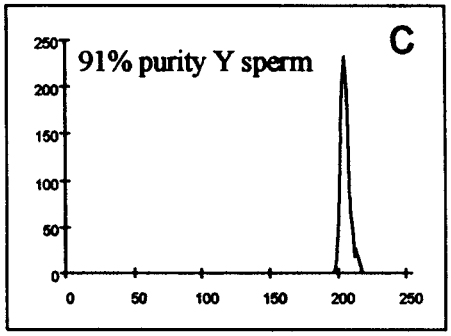
Bovine sperm



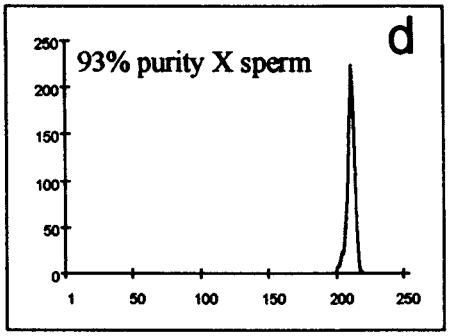
Orientation



DNA-content

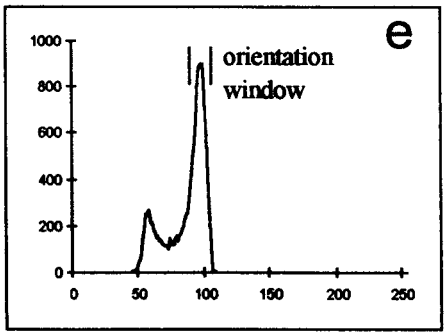


DNA-content

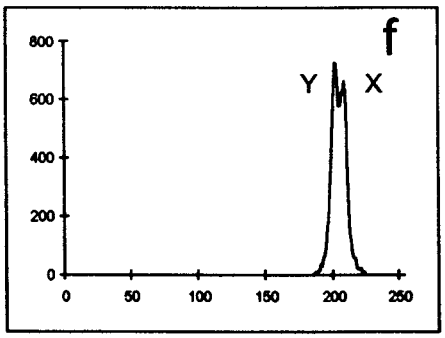


DNA-content

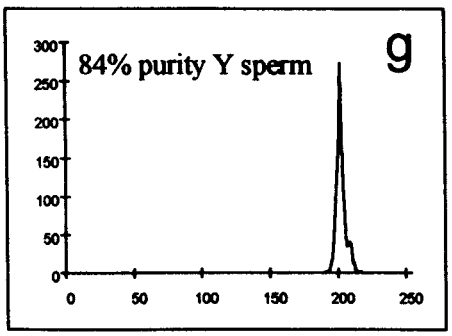
Porcine sperm



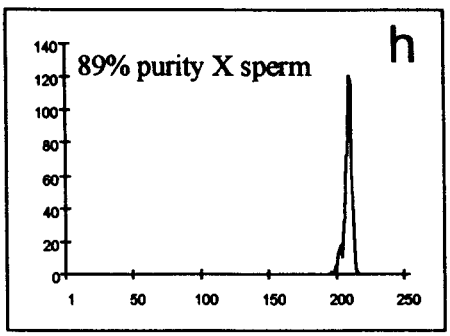
Orientation



DNA-content



DNA-content



DNA-content

Figure 1.

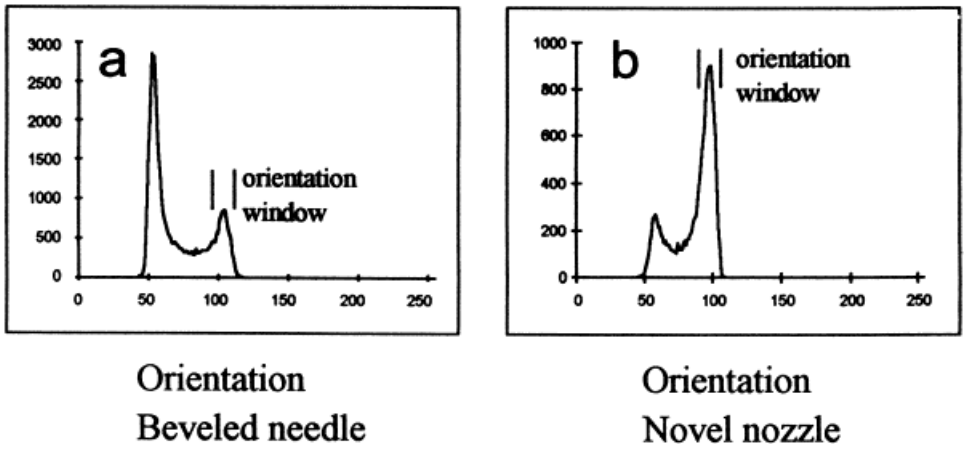


Fig. 2. Orientation histograms of boar intact sperm measured with the beveled needle system (**a**) or with the novel nozzle (**b**). The novel nozzle has a superior orientation performance.

procedure (standard nozzle, beveled needle). The proportion of oriented bull sperm for bull was over 50%, and sorting purities were high (about 88%), showing that analysis and sorting accuracy was maintained. The purities obtained were not different ($P = 0.05$) from those previously published (90.3% X-sperm, and 91.6 % Y-sperm, n = 11) using the modified EPICS 750 series flow cytometer/cell sorter with a beveled needle for sperm orientation. Sorting for that study was performed to obtain offspring after deep uterine artificial insemination with X- and Y-bearing bovine sperm (Seidel et al., 1997).

Also boar sperm, which have less difference in X-Y DNA-content than bull sperm (3.6% vs. 3.8%), were sorted into X and Y bearing sperm populations with high purities. The actual rate of sperm deflected and collected as a putative X and Y population (200 per sec, bull sperm; 180 per sec, boar sperm) were on average twice that obtained using only the beveled needle for orientation.

The numbers presented in this paper for proportions of oriented sperm obtained with the new nozzle average about 55%. However, Fig. 2 shows that part of the sperm population are almost oriented, their signals fall just outside the orientation window. The proportion of oriented sperm would be about 75% if this part could also be used for forward (0°) fluorescence analysis. This fact together with the discussed possibility of higher sample rates would lead to a large or increase in efficiency.

Fig. 1. Results of a bovine and porcine X-sperm and Y-sperm sorting experiment using the novel nozzle. **a**, **e:** Orientation histograms, only sperm with fluorescence signals within the presented window are used for "DNA" analysis. **b**, **f:** DNA histograms, both bovine and porcine histograms show a clear X-Y separation. **c**, **d**: Reanalysis histograms of bovine sorted Y sperm (c) and X sperm (d), both are sorted with high purities. **g**, **h:** Reanalysis histograms of porcine sorted Y sperm (g) and X sperm (h). They also are sorted with high purities.

The more than two fold increase in yield of sorted X- and Y-chromosome bearing sperm plus the prospect of a further increase will make artificial insemination with sexed sperm more feasible. The gender preselection procedure will be less limited to IVF and embryo transfer or surgical insemination allowing a wider application of sexed semen in the livestock industry. In recent months the new nozzle has been fitted and adapted to a high-speed cell sorting system (MoFlo; Cytomation Inc., Ft. Collins, CO). Preliminary results show that the new nozzle amplifies the sorting capability so as to be capable of even greater increases in sorting efficiency (Johnson et al., 1998).

ACKNOWLEDGMENTS

We thank Paul G. Graninger, Diane Coch, and Russ Lange for expert animal management.

REFERENCES

Cran DG, Johnson LA, Polge C. 1995. Sex preselection in cattle: a field trial. Vet Rec 136:495–496.

Cran DG, McKelvey WAC, King ME, Dolman DF, McEvoy TG, Broadbent PJ, Robinson JJ. 1997. Production of lambs by low dose intrauterine insemination with flow cytometrically sorted and unsorted semen. Theriogenology 47:267.

Catt SL, Catt JW, Gomez MC, Maxwell WMC, Evans G. 1996. The birth of a male lamb derived from an in vitro matured oocyte fertilized by intra-cytoplasmic injection of a single presumptive "male" sperm. Vet Rec 139:494–495.

Fugger EF, Black SH, Keyvanfar K, Schulman JD. 1998. Birth of normal daughters after MicroSort sperm separation and intrauterine insemination, in-vitro fertilization, or intracytoplasmic sperm injection. Human Reprod 13:2367–2370.

Johnson LA, Pinkel D. 1986. Modification of a laser-based flow cytometer for high resolution DNA analysis of mammalian spermatozoa. Cytometry 7:268–273.

Johnson LA, Flook JP, Look MV. 1987. Flow cytometry of X- and Y-chromosome bearing sperm for DNA using an improved preparation method and staining with Hoechst 33342. Gamete Research. 17:203–212.

Johnson LA, Flook JP, Hawk HW. 1989. Sex preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 41:199–203.

Johnson LA. 1991. Sex preselection in swine: Altered sex ratios in offspring following surgical insemination of flow sorted X- and Y-bearing sperm. Reprod Domest Anim 26:309–314.

Johnson LA, Welch GR, Garner DL. 1994. Improved flow sorting resolution of X- and Y- chromosome bearing viable sperm separation using dual staining and dead cell gating. Cytometry 17(suppl 7):83.

Johnson LA. 1995. Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: a review. Reprod Fertil Dev 7:893–903.

Long CR, Rath D, Welch GR, Schreier LL, Dobrinsky JR, Johnson LA. 1998. In vitro production of porcine embryos from semen sorted for sex with a high speed cell sorter: comparison of two fertilization media. Theriogenology 49:363.

Pinkel D., Gledhill BL, Van Dilla MA, Stephenson D, Watchmaker G. 1982. High resolution DNA measurements of mammalian sperm. Cytometry 3:1–9.

Rath D, Johnson LA, Dobrinsky JR, Welch GR, Niemann H. 1997. Production of piglets preselected for sex following in vitro fertilization with X and Y chromosome-bearing spermatozoa sorted by flow cytometry. Theriogenology 47:795–800.

Rens W., Welch GR, Houck DW, Van Oven CH, Johnson LA. 1996. Slit-scan flow cytometry for consistent high resolution DNA analysis of X- and Y-chromosome bearing sperm. Cytometry 25:191–199.

Rens W., Welch GR, Johnson LA. 1998. A novel nozzle for more efficient sperm orientation to improve sorting efficiency of X- and Y-chromosome bearing sperm. Cytometry 33: in press.

Seidel Jr GE, Allen CH, Johnson LA, Holland MD, Brink Z, Welch GR, Graham JE, Cattell MB. 1997. Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa. Theriogenology 48:1255–1265.

Steel GD, Torrie JH. 1960. Principals and procedures of statistics. New York: McGraw-Hill Book Company, Inc.

Welch GR, Houck DW, Johnson LA. 1994. Fluidic and optical modifications to a FACS IV for flow sorting of X- and Y-chromosome bearing sperm based on DNA. Cytometry 17(suppl. 7):74.